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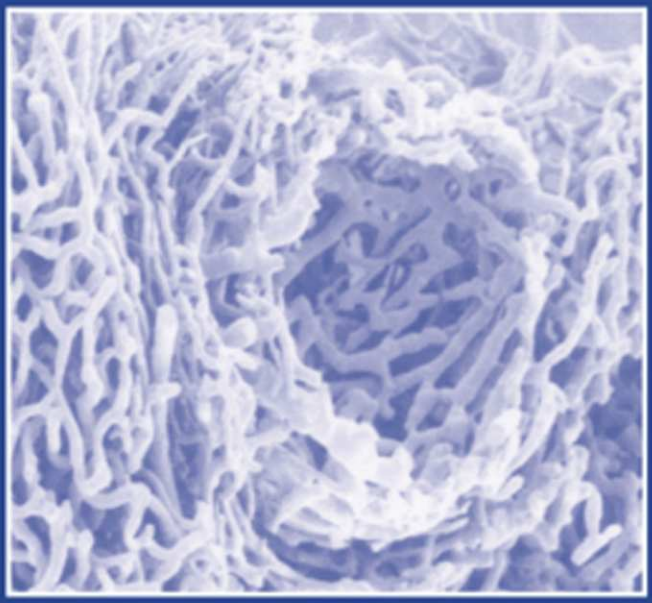
A SURVEY OF CELL BIOLOGY

Edited by
Kwang W. Jeon



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Front cover photograph: (c) A freeze fractured cast, showing the medulla containing large vessels. (For more details, see Chapter 4, Figure 23C)

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Thymic Nurse Cells: A Microenvironment for Thymocyte Development and Selection

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Thymic nurse cells (TNCs) represent a unique microenvironment in the thymus for MHC restriction and T cell repertoire selection composed of a cortical epithelial cell surrounding 20–200 immature thymocytes. TNCs have been isolated from many classes of animals from fish to humans. Studies performed using TNC lines showed that TNCs bind viable $\alpha\beta$TCR^{low} CD4^{+}CD8^{+}CD69^{-} thymocytes. A subset of the bound cells is internalized, proliferates within the TNC, and matures to the $\alpha\beta$TCR^{high} CD4^{+}CD8^{+}CD69^{+} stage, indicative of positive selection. A subset of the internalized population is released while cells that remain internalized undergo apoptosis and are degraded by lysosomes within the TNC. A TNC-specific monoclonal antibody added to fetal thymic organ cultures resulted in an 80% reduction in the number of thymocytes recovered, with a block at the double positive stage of development. Together these data suggest a critical role for TNC internalization in thymocyte selection as well as the removal and degradation of negatively selected thymocytes. Recent studies have shown that in addition to thymocytes, peripheral circulating macrophages are also found within the TNC complex and can present antigens to the developing thymocytes. These circulating macrophages could provide a source of self-antigens used to ensure a self-tolerant mature T cell repertoire. A reduction in TNC numbers is associated with a variety of autoimmune diseases including thyroiditis and systemic lupus erythematosis.

KEY WORDS: Thymic nurse cell, T cell development, MHC restriction, Macrophage, Neuroendocrine, Autoimmunity.

I. Introduction

The thymus is the primary lymphoid organ involved in the differentiation of T lymphocytes. Interactions between developing thymocytes and various cells of the thymic stromal component are critical to both thymopoiesis and development of thymic architecture. The thymus is distinct from secondary lymphoid organs due to its embryonic origin. The thymus is ectodermal and endodermal in origin, explaining the abundance of epithelial cells within the thymic stroma, whereas secondary lymphoid organs are derived from mesoderm (Owen and Jenkinson, 1984). In mice, thymic cortical epithelial cells are derived from the ectodermal branchial cleft, while whereas that medullary epithelium originates from endodermal cells of the third pharyngeal pouch (Weissman, 1985). The organization of epithelial cells in the thymus is distinct from that in other nonlymphoid organs. In most organs, epithelial cells form a sheet of cells positioned on a basement membrane, but in the thymus epithelial cells form a three-dimensional (3-D) sponge-like network of elongated cytoplasmic extensions interconnected by desmosomes (van Ewijk *et al.,* 2000). This sponge-like configuration facilitates the migration of developing thymocytes through the stroma, while enhancing intimate cell–cell interactions between lymphocytes and stromal components, which include distinct epithelial cell types (van Ewijk, 1988, 1991) as well as fibroblasts and hematopoietic cells derived macrophages and dendritic cells (Boyd *et al.,* 1993). The conformation of the thymic stroma creates unique microenvironments for development, which are defined by distinct structural and functional features (van Ewijk, 1991).

The intrathymic developmental pathways that produce mature self-tolerant CD4 and CD8 single positive (SP) T cells from immature CD4 CD8 T cell antigen receptor (TCR) triple negative (TN) precursors have been well documented (Robey and Fowlkes, 1994; Shortman and Wu, 1996). T cell precursors migrate from the fetal liver or the bone marrow and enter the thymus at the corticomedullary junction (Lind *et al.,* 2001). The initial immigrants are multipotent TN $CD44^+CD25^-$ cells that migrate toward the subcapsular cortex while up-regulating CD25 and down-regulating CD44. Between the corticomedullary junction and the subcapsular cortex these cells differentiate from TN $CD44^+CD25^-$ to an intermediate TN $CD44^+CD25^+$ stage and ultimately to the TN $CD44^-CD25^+$ phenotype when they initiate TCR γ-, δ-, and β-gene rearrangements. TN $CD44^-CD25^+$ pre-T cells that productively rearrange the TCR β locus and express the pre-TCR/CD3 complex then rearrange the TCR α-gene, proliferate, and differentiate to the triple positive (TP) $CD4^+CD8^+\alpha/\beta TCR^{low}$ stage. The loss of CD25 expression and the prohibition of further TCR β rearrangements accompany the TN to TP transition. The affinity of the interaction between α/β TCR on TP thymocytes and MHC molecules on antigen-presenting cells of the thymic stroma mediates positive and negative selection processes that determine the mature T cell repertoire. Positive selection ensures that only T cells, expressing TCR that is self-MHC restricted, receive a survival signal. The remaining cells die by neglect. Negative selection

leads to deletion of cells that express high affinity TCRs, which bind strongly to self-peptide/MHC complexes. This process ensures that the mature T cell repertoire is self-tolerant. The binding of TCR to either MHC class I or MHC class II determines whether the particular TP cell down-regulates CD4 or CD8, respectively, and becomes a single positive cell (SP).

Cells that survive positive and negative selection mature to the SP stage and appear to reside primarily in the medulla, or they exit the thymus to become functional peripheral T cells (Fig. 1). Epithelial cells of the thymic cortex form intimate associations with both TN and TP thymocytes (Ceredig *et al.,* 1983; Fowlkes *et al.,* 1985; Scollay and Shortman, 1985; van Ewijk *et al.,* 1981). These interactions are important in both the expansion and differentiation of a variety of thymocyte subpopulations. Thymic epithelial cells (TECs) have been implicated in both positive and negative selection, but it is not clear whether the same population of epithelial cells is responsible for both types of selection. A considerable effort is now underway to determine the intrathymic location and to define the function of individual subpopulations of TECs in effecting T cell development. This review will focus on one of those stromal components, the thymic nurse cell (TNC). We will discuss the work done to characterize and localize this unique multicellular complex composed of cortical epithelial cells, with internalized thymocytes and macrophages. In addition, we will review the data on TNC function derived from both *in vivo* characterization and studies done *in vitro* using isolated TEC lines. Finally, we will address the apparent association of TNCs with autoimmunity.

II. Characteristics of Thymic Nurse Cells (TNCs)

A. Isolation and Characterization

Wekerle and Ketelson in 1980 were the first investigators to identify the multicellular complex in mice, which they named thymic nurse cells (Wekerle and Ketelsen, 1980; Wekerle *et al.,* 1980). Using enzymatic digestion of the thymus with a mixture of collagenase and trypsin, followed by a series of sedimentation steps through layers of fetal bovine serum, they were able to enrich for TNCs. In their initial studies, TNCs were described as keratin-expressing cells containing several thymocytes completely enclosed within specialized cytoplasmic vacuoles (Fig. 2). The number of thymocytes enclosed within an individual TNC varies from 2 to 200 (Andrews and Boyd, 1985; de Waal Malefijt *et al.,* 1986; Ezaki *et al.,* 1991; Wekerle *et al.,* 1980). In our hands, the number of TNC complexes that can be isolated per thymus as well as the number of thymocytes internalized by each epithelial cell vary greatly depending on the strain of mouse used, the age at isolation, and the health of the mouse (M. Pezzano and J. C. Guyden, unpublished results). On average, the yield of TNCs/mouse thymus is approximately 2×10^5, each containing from 7 to 50 thymocytes. This number may be an underestimate

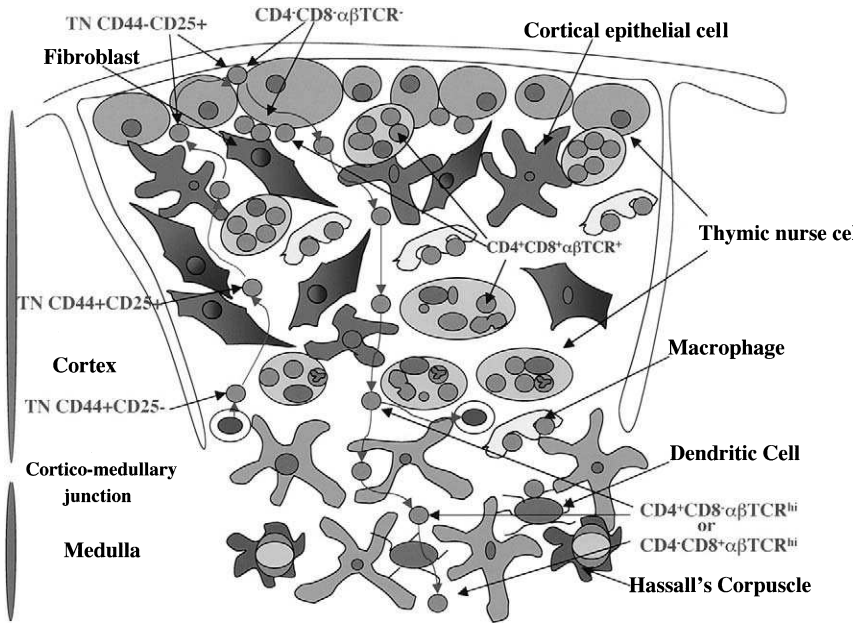

FIG. 1 Schematic representation of T cell migration and interactions with thymic stromal cells during development. The thymus is made up of a complex three-dimensional network of cell types including macrophages, fibroblasts, dendritic cells, and epithelial cells. Complex cell–cell and cytokine interactions between developing thymocytes and this network of stromal cells regulate development of the thymus as well as thymocyte proliferation and selection. Developing thymocytes interact with cells of the thymic stroma to create a repertoire of mature T cells that is both MHC restricted and self-tolerant. This differentiation pathway involves the induction of apoptosis and removal of cells that have the potential to attack normal uninfected cells of self. Immunologists have been able to determine the sequence of events that leads to the maturation of T cells using the expression pattern of CD4, CD8, and $\alpha\beta$TCR as well as other key T cell surface proteins. Early lymphoid immigrants enter the thymus at the corticomedullary junction and are called triple negative cells because they do not express CD4, CD8, or $\alpha\beta$TCR on their cell surface. These cells migrate to the subcapsular cortex and begin to rearrange their antigen receptor genes to express an immature TCR (pre-T$\alpha\beta$TCR) and are then termed double negatives (red arrows show the migration path of thymocytes through the thymus). Cells that make functional rearrangements of the TCR β chain proliferate and rearrange the α chain while turning on both CD4 and CD8. The cells at this stage are called triple positives. After MHC restriction (the process that removes potentially autoreactive thymocytes and allows thymocytes that can recognize foreign antigen on the surface of self-cells to survive), mature T cells are visible within the medulla. Mature T cells express a functional $\alpha\beta$TCR and either CD4 or CD8 and are termed single positive cells. Single positives then exit the thymus through blood vessels at the corticomedullary junction and enter the peripheral T cell pool as functional naive cells. (See also color insert.)

of the actual number of complexes found *in vivo,* given the fragile nature of the TNC complex. Initial investigators were excited because TNCs were also shown to express both class I and class II MHC antigens on their cell surface as well as on the surface of the vacuoles surrounding internalized thymocytes (de Waal Malefijt *et al.,* 1986; Wekerle and Ketelsen, 1980; Wekerle *et al.,* 1980). The

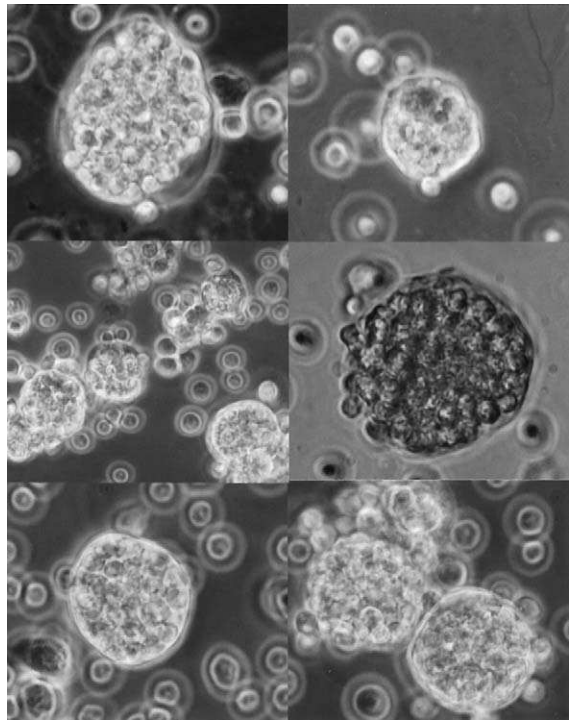

FIG. 2 A collage of light micrographs of a freshly isolated mouse thymic nurse cells. Magnification ×200. TNCs were isolated using combined collagenase/trypsin digestion, followed by enrichment using a 1*g* fetal bovine serum sedimentation gradient. Thymic nurse cells have a very unique morphology when recovered from the thymus. Several thymocytes are visible within the cytoplasm of each thymic nurse cell. Its uniqueness caused many scientists to question whether the thymocytes were actually inside of the epithelial cell or tightly bound to the cell surface. (See also color insert.)

expression of membrane class II MHC antigens is atypical for epithelial cells. The expression of class II antigens is generally thought to be restricted to cells of the immune system. The expression of MHC molecules on the surface of the vacuoles surrounding internalized thymocytes implies a role for TNCs as antigen-presenting cells forming a unique microenvironment within the thymus to regulate thymocyte selection. Studies from our laboratory have shown that antibodies to MHC class I and class II can prevent the TNC-induced rescue of TP thymocytes from apoptosis (Pezzano *et al.,* 1995). These data support the hypothesis that TNCs are critical to both MHC restriction and T cell repertoire selection.

Subsequent to their discovery in mice, TNCs were isolated from the thymus of fish (Flano *et al.,* 1996), frogs (Holtfreter and Cohen, 1987), chickens (Boyd *et al.,* 1984), sheep, pigs, rats (Ezaki *et al.,* 1991), and humans (Ritter *et al.,* 1981; van de Wijngaert *et al.,* 1983). In both mice and rats TNCs express thymic cortical specific markers including ER-TR4 and Th-3 (Adkins *et al.,* 1988; Defresne *et al.,*

1994; Hirokawa *et al.,* 1986; van Vliet *et al.,* 1984; Whitlock *et al.,* 1987). TNCs are not recognized by medullary-specific monoclonal antibodies (mAbs) such as ER-TR5 and Th-4 nor are they recognizable by mAbs specific to macrophages (ED1, ED2, Mac-1, Mac-3), fibroblasts, or thymocytes (Ezaki *et al.,* 1991; van Vliet *et al.,* 1984). TNCs have been shown in a number of studies to express the thymocyte marker Thy-1 (Ezaki *et al.,* 1991). Together these data verify that TNCs are epithelial cells and suggest, on the basis of their expression of cortical specific markers, that they reside in the cortex of the thymus. Because of the complex structure of the thymus, it was difficult to demonstrate the existence of TNC complexes *in vivo*. It is unlikely that the round shape observed *in vitro,* after dissociation from the thymus, is representative of their actual conformation within the thymus. As a result, their exact location within the thymus was impossible to determine without TNC-specific immunological reagents.

A number of investigators initiated studies to determine if TNCs represent an artifact that results from incomplete enzymatic digestion of tightly bound cells during their isolation from the thymus (Kyewski and Kaplan, 1982; Toussaint-Demylle *et al.,* 1990), or if they represent an *in vivo* structure consisting of thymocytes completely enclosed within the TEC membrane. Their existence was verified *in vivo* using EM studies. Micrographs of TNCs isolated from the thymus of all of the species studied appeared to show thymocytes completely surrounded by a membrane within the cytoplasm of a cell containing one large nucleus (Ritter *et al.,* 1981; van de Wijngaert *et al.,* 1983; Wekerle and Ketelsen, 1980; Wekerle *et al.,* 1980). Although it is difficult to show the intact structure of TNCs *in vivo* using frozen sections of the thymus because of the very high density of cells within the thymic cortex, visualization of the entire membrane surrounding engulfed thymocytes has been presented. These experiments were done using the human thymus tissue and staining with antibodies against keratins or MHC proteins (Dipasquale and Tridente, 1991; Ritter *et al.,* 1981). Transmission electron micrographs (TEMs) of TNCs reveal a prominent nucleus within the cytoplasm containing enclosed thymocytes (Fig. 3). Cytoplasmic organelles, mitochondria, Golgi, and lysosomes have also been described within the membrane of TNCs (Penninger *et al.,* 1994). Other investigators used biochemical techniques to determine the integrity of this unusual structure (Wekerle *et al.,* 1980). Extended treatment of TNCs with trypsin was used to determine the structural relationship between trapped thymocytes and TNCs. Extensive treatment with trypsin and collagenase does not dissociate the cells within the complex (M. Pezzano and J. C. Guyden, unpublished data). Further, the thymocyte subset within TNCs is inaccessible to thymocyte-specific antibodies before fixing and permeabilization with detergent or acetone (Li *et al.,* 1992; Wekerle and Ketelsen, 1980).

Additional support for cytoplasmic enclosure of thymocytes by TNCs was provided when the structure could be reconstituted using TNC lines (Fujiwara *et al.,* 1990; Itoh *et al.,* 1988; Nakashima *et al.,* 1990; Nishimura *et al.,* 1990; Pezzano *et al.,* 1991; Philp *et al.,* 1993; Tatsumi *et al.,* 1990). The generation of TNC lines

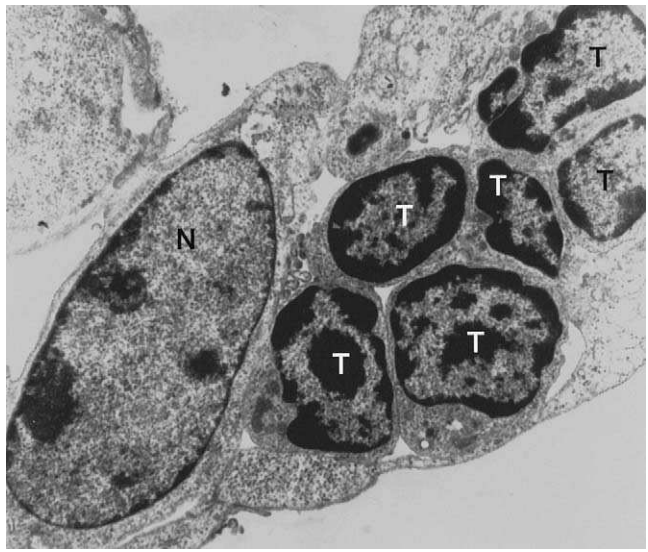

FIG. 3 Transmission electron microscopy of an internalized thymocyte. Thymocytes were centrifuged onto monolayers of tsTNC-1 cells (an SV40-immortalized TNC line) and incubated overnight before being prepared for examination. The micrograph shows six internalized thymocytes (labeled with T) within the TNC. Cytoplasmic organelles and the nucleus (N) of the TNC are distinguishable. Magnification ×5600.

was important because prior to their development, the formation of this unique multicellular complex in culture had not been reported. Freshly isolated TNCs attach themselves to the bottom of tissue culture plates and release their enclosed thymocytes, but the subsequent uptake of thymocytes by freshly isolated TNCs has not been reported. TNC cell lines were able to internalize thymocytes that were added separately to *in vitro* cultures. Data obtained from TEM and antibody-staining experiments have shown multicellular complexes resulting from TNC internalization of added thymocytes. The internalization event has also been visualized using long-term video microscopy (Philp *et al.,* 1993). In this presentation, internalization is defined as membrane sealed or complete separation from the extracellular environment.

B. TNC Functions as Studied in Cell Lines

The internalization of thymocytes by TNCs was captured through the addition of freshly isolated thymocytes to cultures containing cells derived from the immortalization of thymic nurse cells (Gao *et al.,* 1993; Hiramine *et al.,* 1990, 1996; Itoh *et al.,* 1988; Nakashima *et al.,* 1990; Pezzano *et al.,* 1991; Philp *et al.,* 1993). Prior to those reports, it was difficult to determine the authenticity of these cell lines

because no TNC-specific antigens had been defined. At that time the only TNC-specific characteristic observed was their ability to engulf immature thymocytes. Earlier, Jason and Janeway (1984) produced short-term cultures of thymic epithelial cells with characteristics similar to TNCs. These cells were maintained in culture for approximately 6 weeks before fibroblast overgrowth. Although some of the cells in their cultures were able to engulf thymocytes, they were not termed thymic nurse cells. Similarly, Nakashima *et al.* (1990) isolated a long-term thymic epithelial line (TEL-2) from BALB/c mice that forms TNC-like complexes upon incubation with normal mouse thymocytes. The cells of this line expressed none of the T-cell-specific antigens and were Mac-1^-. TEL-2 cells expressed the 6C3 antigen, a cortical marker, and were MHC class I positive. Cell surface MHC class II antigen was not detectable. Again, these cells were not referred to as TNCs. Three long-term lines were established and characterized as thymic nurse cells (Itoh *et al.,* 1988; Nishimura *et al.,* 1990; Pezzano *et al.,* 1991). Itoh *et al.* (1988) isolated an epithelial cell clone (IT-79MTNC3) from a spontaneous thymic tumor in a BALB/c mouse. These cells expressed Ia antigens only in the presence of γ-interferon. The nature of class I MHC antigen expression in these cells was not presented. IT-79MTNC3 cells support the proliferation of fetal thymocytes in the presence of recombinant interleukin-2 (rIL-2). IT-79MTNC3 cells develop complexes with thymocytes that are similar to those of freshly isolated TNCs. TEM studies showed thymocytes to be tightly bound to the surface of IT-79MTNC3 cells, but internalized thymocytes were not detected.

Cells from another nurse cell line, termed B/c TEC-L1 (Hiramine *et al.,* 1990), were shown to take up PNA^+ lymphocytes in culture. PNA is exclusively expressed on triple positive thymocytes (Philp *et al.,* 1993). Complete internalization of thymocytes by B/c TEC-L1 cells was not described. Another TNC line was developed from the infection of freshly isolated TNCs (from C57BL/6 mice) with the SV40 virus (Pezzano *et al.,* 1991). Cells of two identical clones were shown to internalize $\alpha\beta TCR^{lo}CD4^+CD8^+$ thymocytes exclusively *in vitro* (SVT-MP5 and SVT-II2) (Li *et al.,* 1992). Cells from both cell lines expressed cytokeratins, class I and class II MHC antigens. The long-term cultures of tsTNC-1 cells, another TNC line generated through SV40 transformation with freshly isolated thymocytes, resulted in the maturation of $\alpha\beta TCR^{lo}CD69^-$ triple positives into $\alpha\beta TCR^{hi}CD69^{hi}$ cells (Pezzano *et al.,* 1996). SV40-derived TNC lines were also shown to express the neuroendocrine marker A2B5, which was previously shown to be a characteristic of freshly isolated TNCs. A2B5 has been shown to colocalize in TNCs along with the neuropeptides oxytocin, arginine-vasopressin, and their associated neurophysins (Geenen *et al.,* 1988; Pezzano *et al.,* 1991).

C. Intrathymic Location

Finding the exact intrathymic location of TNCs is important because this information would aid in the determination of the subset of thymocytes that interacts with

TNCs. Such information would subsequently shed light on TNC function during thymocyte development. This question was first addressed in a study by Kyewski and Kaplan (1982). They immersed the entire thymus in fluorescein isothiocyanate (FITC) for increasing time periods and analyzed the incorporation of fluorescein in TNC thymocytes versus free thymocytes. At each time point from 15 to 60 sec there was a higher percentage of fluoresceinated thymocytes associated with TNCs and an increase in the number of TNCs. They interpreted these results to demonstrate the presence of TNCs near the outer cortex of the thymus. The open-ended nature of these results did not define the limits of the compartment within the thymic cortex that house TNCs. However, in our laboratory, subsequent staining experiments using the TNC-specific monoclonal antibody ph91 revealed TNCs to be scattered throughout the cortex from the subcapsular region to the corticomedullary junction with the greatest density of staining occurring in the outer cortex (Pezzano *et al.,* 1998).

In a number of early studies it was demonstrated that TNCs react with the cortical-specific mAb ER-TR4 (Defresne *et al.,* 1994). ER-TR4 identified epithelial cells defined as "Type 1," which were localized throughout the cortex and to a limited extent in the outer medulla. It is yet to be determined whether TNCs represent a subclass of this Type 1 epithelial cell or whether they are all TNCs at various stages of interaction with thymocytes. A similar staining pattern was observed for expression of A2B5 (Haynes *et al.,* 1983) as well as antineuropeptide antibodies (Geenen *et al.,* 1988). TNCs are defined as neuroendocrine cells by their expression of both A2B5 and a number of neuropeptides including oxytocin arginine-vasopressin and their associated neurophysins (Geenen *et al.,* 1988; Pezzano *et al.,* 1991). This characteristic appears to be unique to TNCs within the thymus. Although colocalization studies were not performed, the similarity in staining pattern provides strong evidence that the various antibodies are identifying the same subset of epithelial cells. Taken together these data suggest that TNCs are localized throughout the cortex and possibly to a limited extent in the medulla. This intrathymic location places them in a key location for interacting with immature thymocytes during the TN and TP stages of thymocyte development, which represent critical windows for thymocyte proliferation, MHC restriction, and TCR repertoire selection.

D. The TNC-Interactive Thymocyte Subset

One way to localize TNCs within the thymus is to define the thymocyte subset(s) enclosed within the cytoplasm of TNCs. Several coculture studies defined the TNC-internalized subset to be triple positive (Holtfreter and Cohen, 1987; Li *et al.,* 1992; Nakashima *et al.,* 1990). However, conflicting evidence exists. Some reports show the TNC-interactive subset to have the triple negative phenotype (Gao *et al.,* 1993; Itoh *et al.,* 1988). One TNC clone, TNCR3.1, establishes multicellular complexes *in vitro* with triple negative thymocytes and supports growth

and maturation of its interactive subset to the triple positive window of development through the $CD3^{-}CD4^{+}$ intermediate pathway. Similarly, IT-79MTNC3, another mouse TNC clone, supports growth and maturation of its interactive subset from the triple negative window of development to the $CD3^{-}CD4^{+}$ intermediate stage. Internalization of thymocytes was not detected in IT-79MTNC3 cocultures. Support for the triple positive phenotype of TNC thymocytes has been obtained through analysis of thymocytes enclosed within freshly isolated TNCs (Ezaki *et al.,* 1991; Kyewski and Kaplan, 1982). Such conflicting data create difficulty in assigning an exact thymic location to TNCs. Multiple types of epithelial cells may be able to form TNC-like complexes with developing thymocytes at distinct developmental stages. Alternatively, TNCs may interact with thymocytes at the TN stage and drive development to the late TP stage or possibly even the SP stage *in vivo*.

It appears likely that both development of thymocytes and the development of the epithelial component of the thymic microenvironment follow the same pattern and are interdependent on each other. The most immature thymocytes enter the thymus at the corticomedullary junction and then migrate toward the subcapsular cortex (Lind *et al.,* 2001). Along the way they differentiate from TN $CD44^{+}25^{-}$ cells to TN $CD44^{+}CD25^{+}$ and ultimately to TN $CD44^{-}CD25^{+}$cells as they reach the subcapsular cortex. Differentiation of thymocytes to this stage and interaction with the epithelial component of the thymic stroma are critical for the proper development of the epithelial cells as well as the thymocytes. van Ewik *et al.* (2000) reported major structural abnormalities in the epithelial component of the thymus in human CD3ε transgenic mice, where thymocyte development is blocked at the TN $CD44^{+}25^{-}$ stage. In these animals, the epithelial architecture of the thymus does not develop the proper 3-D organization, but remains as two-dimensional (2-D) sheets parallel to the thymic capsule. In RAG^{null} animals, where development arrests at the later TN $CD44^{-}CD25^{+}$ stage, the 3-D organized cortical epithelial network develops normally. They also report an abundance of TNC-like complexes, in RAG^{null} animals, that were completely absent in the human CD3ε transgenic mice. When bone marrow from RAG^{null} animals is injected into the CD3ε transgenic mice, a normal 3-D organization of the cortical epithelial network is restored and TNCs are formed. It is important to note that no TNC-specific antibodies were used to verify that the complexes identified using TEM were TNCs. A follow-up to this study using either A2B5 or the TNC-specific monoclonal antibody PH91 (Pezzano *et al.,* 1998) would allow clarification of this point.

A recent ultrastructural study suggests that at least three different types of TNC with distinct functions may exist in the thymus (Brelinska and Warchol, 1997). The TNCs identified in RAG^{null} mice resemble the described type 1 TNC, which are thought to be involved in the early expansion of T cell progenitors, as many TNC complexes in Rag^{null} mice contain dividing lymphoid cells. van Ewijk proposes that the proliferating cells in TNC probably represent TN thymocytes shifting

from the TN $CD44^+CD25^+$ phenotype to the TN $CD44^-CD25^+$ stage (van Ewijk *et al.,* 2000). The TN $CD44^-CD25^+$ phenotype is subject to β selection (Fehling and von Boehmer, 1997), which suggests that type 1 TNC may play a critical role in regulating this crucial checkpoint in thymopoiesis. TNCs harbor proliferating thymocytes at both the TN and DP developmental stages (Brelinska, 1989; Doi *et al.,* 1991; Ezaki *et al.,* 1991; Pezzano *et al.,* 1996). There appears to be a trend in the literature to classify any epithelial cell, which has the ability to associate closely with thymocytes, as a TNC. This approach has led to conflicting reports about the role of TNC in thymocyte development. Because many of these cells are not well defined, much work is needed in future studies to define different classes of TNCs on the basis of the thymocyte subset with which they interact.

Alternatively, other distinguishing characteristics of the epithelial cells themselves, such as keratin or adhesion molecule expression and intrathymic location, must be used to define these important cell–cell interactions. Our studies have focused on the population of cortical epithelial cells that is enriched for, using the methods originally described by Wekerle and Ketelsen (1980). This population of cells appears to associate exclusively with the TP thymocyte subset as well as a limited number of macrophages. This was verified in both reconstitution studies using SV40 immortalized murine TNC lines (Fig. 4, CD4 CD8 staining) as well as with freshly isolated TNC complexes (Fig. 4, CD4 CD8 staining) (Pezzano *et al.,* 1995; Philp *et al.,* 1993). In a recent study using fetal C57BL/6 mice harvested from Day 14 to Day 19 of gestation, we demonstrated that TNC complexes do not appear in the thymus until Day 19 of fetal development. The appearance of TNC complexes in these animals correlated with the appearance of $\alpha\beta$TCR high cells and a small number of CD4 and CD8 SP cells in the thymus, but was subsequent to the appearance of $\alpha\beta TCR^{low}$ $CD4^+CD8^+$ cells (M. Pezzano, unpublished data). These results strongly suggest that *in vivo,* TNC associate only with thymocytes at the TP stage and that the interaction is critical for differentiation to mature phenotypes. We suggest that some of the early studies that demonstrated either TN or SP thymocyte subsets within these TNC complexes were either identifying internalized macrophage populations or variations in staining level associated with the induction of apoptosis. The opposing results obtained with isolated cell lines have initiated considerable controversy. It should be stated, however, that until TNCs containing triple negative thymocytes are described in freshly isolated preparations, the development of epithelial cell lines that interact with triple negative thymocytes may represent a thymic stromal cell population that is distinct from TNCs. For future studies of TNC function in thymocyte selection, it will be critical to classify these other cell types that interact with more immature thymocyte subsets as a TEC distinct from TNCs. It must be stated that the studies described above were performed using mammalian cells. The cell-surface phenotype of the thymocyte population within TNCs isolated from animals in other classes varies significantly from those found in mice, rats, and humans.